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**METHOD FOR ESTIMATION OF
VITAMIN D IN FOODSTUFFS**

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**BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002**

Indian Standard

METHOD FOR ESTIMATION OF VITAMIN D IN FOODSTUFFS

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Indian Standard

METHOD FOR ESTIMATION OF VITAMIN D IN FOODSTUFFS

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 30 November 1970, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Vitamins are required to be assessed in a large number of foodstuffs, such as dairy products, animal feeds, processed cereals and other foodstuffs. Moreover, different methods of vitamin assay are used in different laboratories. Therefore, with a view to establishing uniform procedures and also for facilitating a comparative study of results, ISI is bringing out a series of standards on vitamin assays. These would include chemical and microbiological methods, wherever applicable.

0.3 The method is mainly applicable to foods fortified with vitamin D or foods with higher vitamin D content.

0.3.1 Although biological assay of vitamin D gives better results, chemical method has been recommended keeping in view the facilities available in the country.

0.4 In formulation of this standard, considerable assistance has been derived from Pharmacopoeia of the United States of America, XVII. 1965.

0.5 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

1. SCOPE

1.1 This standard prescribes a method for the estimation of vitamin D in foodstuffs.

2. QUALITY OF REAGENTS

2.1 Unless specified otherwise, pure chemicals shall be employed in tests

*Rules for rounding off numerical values (*revised*).

and distilled water (*see* IS:1070-1960*) shall be used where the use of water as a reagent is intended.

NOTE— 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the result of analysis.

3. PREPARATION OF THE ASSAY SAMPLE

3.0 The technique used for preparing the material for the analysis is mostly common to every vitamin determination. It should be ensured that the sample taken for the assay is representative of the whole, and any deterioration of the vitamin to be examined is prevented.

3.1 Powders and liquids should be mixed thoroughly until homogeneity is achieved. Dry materials, such as bread, biscuits and grains, should be ground and mixed thoroughly.

3.2 Butter should be melted under constant stirring. Samples from margarine or cheese or other such foods should contain portions of the surface as well as of the interior.

3.3 Wet or fresh material may be minced with a knife or scissors, or homogenized in a blender, if necessary, in the presence of the extracting solvent.

4. METHOD

4.0 Principle—The method is based upon the measurement of colour produced when a solution of vitamin D is treated with antimony trichloride.

4.1 Reagents and Apparatus

4.1.1 Chromatographic Fuller's or Equivalent Earth—Use chromatographic fuller's or equivalent earth having a water content corresponding to not less than 8.5 percent and not more than 9.0 percent of loss on drying.

4.1.2 Solvent Hexane—Redistilled, if necessary, so that when measured in a 1-cm cell at 300 m μ with a suitable spectrophotometer, against air as the blank, the absorbance shall not be more than 0.070.

4.1.3 Ethylene Dichloride—Purified by passage through a column of granular [passing through 75- to 710-micron IS Sieve (*see* IS: 460-1962†)] silica gel (chromatographic grade).

4.1.4 Potassium Hydroxide Solution—Dissolve 730 g of potassium hydroxide in water to make 1 000 ml.

4.1.5 Cotton Seed Oil—Use cotton seed oil that meets the following requirements:

*Specification for water, distilled quality (*revised*).

†Specification for test sieves (*revised*).

Saponify 10 g of the oil and dissolve the unsaponifiable residue in 10 ml of solvent hexane. In a separate container place 0.4 ml of ferric chloride solution (1 in 1000) and 12 ml of 1 in 6000 solution of α , α' -dipyridyl in absolute alcohol; then mix and 5 minutes later read the absorbance in a 1-cm cell at 520 $m\mu$, with a suitable spectrophotometer using absolute alcohol as the blank. Then add 0.2 ml of the solvent hexane solution of the unsaponifiable residue, and after 5 minutes read the absorbance. The difference between the first and the second absorbances shall be not less than 0.125.

4.1.6 Sodium Sulphate—anhydrous.

4.1.7 Sodium Sulphate—decahydrate.

4.1.8 Colour Reagent—Prepare two stock solutions as follows:

Solution A—Empty, without weighing, the entire contents of a previously unopened 113 g bottle of dry, crystalline antimony trichloride into a flask containing about 400 ml of ethylene dichloride. Add about 2 g of anhydrous alumina, mix and filter through filter paper into a clear-glass, glass-stoppered container calibrated at 500 ml. Add ethylene dichloride to make the volume 500 ml, and mix. The absorbance of the solution, measured in a 20-mm cell at 500 $m\mu$ with a suitable spectrophotometer, against ethylene dichloride, should not exceed 0.070.

Solution B—Mix, under a hood, 100 ml of acetyl chloride and 400 ml of ethylene dichloride.

Mix 45 ml of solution A and 5 ml of solution B to obtain the colour reagent. Store in a tight container, and use within 7 days, but discard any reagent in which a colour develops.

4.1.9 Chromatographic Tubes

4.1.9.1 First column—Arrange for descending column chromatography a tube of 2.5 cm (inside) diameter, about 25 cm long, and constricted to 8 mm diameter for a distance of 5 cm at the lower end, by inserting at the point of construction a sintered-glass disc of coarse-porosity or a small plug of glass-wool. The constricted portion may be fitted with an inert, plastic stopcock.

4.1.9.2 Second column—Select a tube that is made up of three sections: (i) a flared top section, 18 mm inside diameter and approximately 14 cm long; (ii) a middle section, 6 mm inside diameter and approximately 25 cm long; and (iii) a tapered constricted lower exit-tube approximately 5 cm long. Insert a small plug of glass-wool in the upper 1-cm portion of the constricted section.

4.1.10 Chromatographic Columns

4.1.10.1 First column—To about 125 ml of iso-octane contained in a screw-capped wide mouth bottle add 25 g of chromatographic siliceous earth, and shake until a slurry is formed. Add, dropwise and with vigorous

mixing, 10 ml of polyethylene glycol 600. Replace the bottle cover and shake vigorously for 2 minutes. Pour about half of the resulting slurry into the chromatographic tube and allow it to settle by gravity. Then apply gentle suction and add the remainder of the slurry in small portions, packing each portion with a 20-mm disc plunger. When a solid surface has formed, remove the vacuum, and add about 2 ml of iso-octane.

4.1.10.2 Second column — Pack the midsection of the tube with 3 g of moderately coarse chromatographic fuller's or equivalent earth with the aid of gentle suction (about 125 mm Hg).

4.1.10.3 Standard preparation — Dissolve about 25 mg of USP or equivalent Ergocalciferol Reference Standard, accurately weighed, in sufficient iso-octane to give a known concentration of about 250 µg per ml. Store in a refrigerator.

On the day of assay, pipette 1 ml of the standard solution into a 50-ml volumetric flask, remove the solvent with a stream of nitrogen and dissolve the residue in, and make to volume with, ethylene dichloride.

5. SAMPLE PREPARATION

5.1 Accurately weigh or measure a portion of the sample to be assayed, equivalent to not less than 125 µg, but preferably about 250 µg of ergocalciferol. If little or no vitamin A is present in the sample, add about 1.5 mg (the equivalent of 3000 USP units) of vitamin A acetate to provide the needed pilot bands in the subsequent chromatography.

Add 2 ml of cotton seed oil, then add a volume of potassium hydroxide solution representing 2.5 ml for each g of the total weight of the sample plus cotton seed oil, but not less than 15 ml; then add 50 ml of alcohol. Reflux vigorously on a steam-bath for 20 minutes, or for 30 minutes for samples weighing more than 5 g. If oil globules are evident indicating incomplete saponification, add 5 ml of potassium hydroxide solution (but not exceeding a total of 100 ml), and heat again. Cool, and transfer the saponified mixture to a 500-ml separator rinsing the saponification flask with a total of 50 ml of water and adding each rinse to the separator. Add about 4.5 g of sodium sulphate decahydrate and 150 ml of solvent hexane. Shake vigorously for 2 minutes. When the aqueous layer has separated, transfer it to a second separator. Extract with two 50-ml portions of solvent hexane, combine the extracts, and discard the aqueous solution.

Wash the combined solvent hexane extracts with 50-ml portions of water until the last portion shows no pink colour on the addition of phenolphthalein indicator solution. Allow the washed extract to stand for 5 minutes, discard any water that separates, and transfer the extract to a 300 ml tall-form beaker containing about 5 g of anhydrous sodium sulphate. Stir for 2 minutes, and decant the solution into a 500 ml, tall-form beaker.

Rinse the sodium sulphate with four 25-ml portions of solvent hexane combining the rinsings with the original extract. Reduce the total volume to about 30 ml by evaporation on a steam-bath, and transfer the concentrate to a small, round-bottom evaporation flask. Rinse the beaker with four 5-ml portions of solvent hexane, adding the rinsings to the flask. With the aid of vacuum in a water-bath at a temperature not exceeding 40°C, or with a stream of nitrogen at room temperature, remove the remaining solvent completely. Dissolve the residue in a small amount of solvent hexane, transfer to a 10-ml volumetric flask, and dilute with solvent hexane to volume to obtain the sample preparation.

6. PROCEDURE

6.0 Solutions of vitamin should be protected from oxygen and actinic light.

6.1 First Column Chromatography—Just as the 2 ml of iso-octane disappears into the surface of the prepared first column, pipette 2 ml of the sample preparation on to the column. As the meniscus of the sample preparation reaches the column surface, add the first of three 2-ml portions of solvent hexane, adding each succeeding portion as the preceding portion disappears into the column. Continue adding solvent hexane in portions of 5 to 10 ml until 100 ml has been added. If necessary, adjust the flow rate to between 3 and 6 ml per minute by application of gentle pressure at the top of the chromatographic tube.

Discard the first 20 ml of effluent and collect the remainder. Examine the column under ultraviolet light at intervals during the chromatography, and stop the flow when the front of the fluorescent and representing vitamin A is about 5 mm from the bottom of the column. (The ultraviolet lamp should provide weak radiation in the 300 m μ region. It is frequently necessary to use a narrow aperture or screen with commercial lamps to reduce the amount of radiation to the minimum required to visualize the vitamin A on the column.)

Transfer the eluate to a suitable evaporation flask, and remove the solvent hexane completely under vacuum at a temperature not above 40°C or with a stream of nitrogen at room temperature. Dissolve the residue in about 10 ml of solvent hexane.

6.2 Second Column Chromatography—Add the solvent hexane solution obtained as directed under first column chromatography on to the second column. Rinse the evaporation flask with a total of 10 ml of solvent hexane in small portions, adding each portion to the second column and allowing it to flow through the column, and discard the effluent. When about 1 ml of the solvent hexane remains above the surface of the column, add 75 ml of benzene and elute with the aid of gentle suction (about 125 mm Hg), collecting the eluate. Evaporate the benzene under vacuum at a temperature not above 40°C or with a stream of nitrogen at room temperature.

6.3 Assay Preparation—Dissolve the residue obtained as directed under second column chromatography in a small amount of ethylene dichloride, transfer to a 10-ml volumetric flask, and make to volume with ethylene dichloride to obtain the assay preparation.

6.4 Colour Development—Into each of three suitable matched colorimeter tubes of about 20 mm inside diameter, and designated 1, 2 and 3 respectively, pipette one ml of the assay preparation. Into tube 1 pipette one ml of the standard preparation, into tube 2 one ml of ethylene dichloride and into tube 3 one ml of a mixture of equal volumes of acetic anhydride and ethylene dichloride. To each tube add quickly, and preferably from an automatic pipette, 5.0 ml of colour reagent and mix. Forty-five seconds after the addition of the colour reagent, determine the absorbances of the three solutions at 500 m μ with a suitable spectrophotometer, using ethylene dichloride as the blank. Similarly, 45 seconds after making the first reading on each solution, determine the absorbances, of the solutions in tubes 2 and 3 at 550 m μ in a similar manner. Designate the absorbances as A^1_{500} , A^2_{500} , A^3_{500} , A^2_{550} and A^3_{550} respectively in which the superscript indicates the number of the tube and the subscript the wavelength.

7. CALCULATION

7.1 Calculate the quantity, in μg of vitamin D in the portion of the sample taken by the following formula:

$$(Cs/C)(Au/As)$$

where

Cs = concentration of vitamin D in μg per ml of the standard preparation,

C = concentration of the sample as g in each ml of the final solution,

Au = value of $(A^2_{500} - A^3_{500}) - 0.67(A^2_{550} - A^3_{550})$ determined from the absorbance observed on the solution from the assay preparation, and

As = value of $A^1_{500} - A^2_{500}$ determined on the solution from the standard preparation.

BUREAU OF INDIAN STANDARDS

Headquarters:

Manak Bhavan, 9 Bahadur Shah Zafar Marg, NEW DELHI 110002

Telephones: 331 01 31, 331 13 75

Telegrams: Manaksanstha
(Common to all Offices)

Regional Offices:

	Telephone
Central : Manak Bhavan, 9 Bahadur Shah Zafar Marg, NEW DELHI 110002	{ 331 01 31 331 13 75
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